

**INHIBITION OF BONE TUMOR FORMATION USING
ANTISENSE cDNA THERAPY**

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Field of Invention

[0001]

The present invention relates to the use of antisense cDNA targeting for the inhibition and/or prevention of tumor growth. More specifically, this invention relates to the use of antisense nucleic acid derived from the antisense cDNA sequence of tubedown-1 for the inhibition and/or prevention of bone tumors, especially osteosarcoma and Ewings Sarcoma family of tumors.

Background

[0002]

Cancer is generally treated with cytoreductive therapies that involve administration of ionizing radiation or chemical toxins that kill rapidly dividing cells. Unfortunately, these therapies are highly toxic to

"Express Mail" mailing label number non-cancer cells and cause severe side effects, such as bone marrow
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[0003]

Osteosarcoma, a bone cancer occurring primarily in teenagers and young adults, affects approximately 2100 individuals yearly in the United States (1). This malignancy accounts for as many as 5% of all childhood malignancies and 60% of all malignant childhood bone tumors (2). Despite radical surgical resection of the primary tumor and aggressive adjuvant chemotherapy, the overall 2-year metastasis-free survival rate approaches only 66%. More than 30% of patients with this disease develop lung metastasis within the first year (3-4). The survival rate among those affected with osteosarcoma has not changed significantly over the past 10 years, despite changes in adjuvant chemotherapy (5).

[0004]

Ewing's Sarcoma is the second most frequent type of bone tumor and Ewing's Sarcoma most often strikes during the second decade of life. Tumors can metastasize to lungs, other bones and to the bone marrow (6-8). Approximately 25% of Ewing's tumor patients present detectable metastatic disease at diagnosis, but it is probable that most patients have micrometastases (9-10). Approximately 40 percent of Ewing's sarcoma patients do not survive. Extrasosseous Ewing's sarcoma variants including the peripheral primitive neuroectodermal tumor (PNET) and the Askin tumor (a thoracic form of Ewing's sarcoma) are more rare (7-8).

[0005]

Chemotherapy using cytotoxic drugs (vincristine, actinomycin-D, cyclophosphamide, etoposide), followed by surgery or radiotherapy is the current treatment regimen for Ewing's sarcomas (6, 10). There is a 50-70% survival rate (5 years) in cases of localized disease. However, when metastatic disease is observed, there is a 19-30% survival rate (5 years)

(10). Moreover, the risk of developing secondary malignancies is approximately 6.7% at 10 years and 43% at 20 years, and relapse 5 years after treatment occurs in approximately 9-16% of cases (7, 9). The intensive radiation and chemotherapy treatments for both osteosarcoma and Ewing's sarcoma tumors are associated with a high degree of toxicity. Thus, new treatments are needed for these types of pediatric bone tumors.

[0006]

Although the genetic alterations involved in Ewing's sarcoma have been identified, understanding how these pathologically modified genetic pathways lead to and support growth of Ewing's tumors have not been completely realized. The isolation, characterization and practical manipulation of additional regulatory molecules which could play a common role in the growth control of Ewing's sarcoma cells may lead to new and improved therapies for the Ewing's family of tumors. Such new therapy would be aimed at altering the signaling pathways which regulate growth of Ewing's tumor cells and could offer alternatives or supplements to the currently available treatments.

[0007]

Furthermore, even less is known about the genetics or growth control of osteosarcomas. The isolation, characterization and practical manipulation of master regulatory molecules which could play a common role in the growth control of bone tumor cells may lead to new and more effective therapies for bone cancer. New treatments aimed at altering the signaling pathways which regulate growth of bone tumor cells offers alternatives or supplements to the exclusive use of radiotherapy, cytotoxic chemotherapeutic drugs and surgery.

[0008]

The inventors have cloned a new gene named tubedown-1 (tbdn-1) which encodes a novel protein associated with an acetyltransferase activity (11). This tubedown-1 protein is highly expressed in primitive bone tumors of mesenchymal and neuroectodermal origin such as osteosarcoma and Ewing's sarcoma. The *in vivo* expression pattern of tbdn-1 suggests it may play a role in regulating endothelial, hematopoietic and bone development (Figure 1) (11). In early myeloid blood cells, blood vessel endothelium and bone, tbdn-1 is expressed at high levels early on and becomes downregulated as these cells mature (11). Tbdn-1 expression distribution during embryogenesis is similar to the expression distribution of FLI-1 (12). In adults, tbdn-1 is restricted to ocular and ovarian blood vessels, bone marrow and atrial endocardium.

[0009]

Tbdn-1 is also expressed highly in Ewing's sarcoma cells, suggesting that it may play a regulatory role in this type of tumor. This finding implies that tbdn-1 may play a regulatory role in these types of bone cancer and perhaps bone cancer in general. Therefore, one approach to the treatment of these sarcomas would be a gene therapy approach aimed to block expression of tbdn-1 in these bone tumors by inducing expression of an antisense tbdn-1 cDNA fragment (ASTbdn-1) which inhibits tumor growth.

Summary of the Invention

[0010]

As described herein, the present invention comprises a method to use tbdn-1 antisense reagents as gene therapy agents for the treatment of

bone tumors and Ewing's sarcoma family of tumors. Antisense-based reagents, such as tbdn-1 antisense construct or biologically stabilized oligonucleotides, or any compound which would elicit the downregulation of tbdn-1 level or activity and the same biological effects as tbdn-1 antisense construct on bone tumor growth in vivo provide valuable alternative or supplemental therapies for bone cancer.

[0011] The antisense cDNA molecules utilized in the present invention generate antisense mRNA of at least 70% complementarity to mRNA produced by a native tubedown-1 gene. Preferred antisense oligonucleotide molecules are selected from the group consisting of SEQ ID NO. 3 and SEQ ID NO. 4. These above-cited antisense oligonucleotides derived from the cDNA sequence of SEQ ID NO. 2, may also be formulated as compositions comprising a safe and effective amount of an antisense oligonucleotide molecule and a pharmaceutically acceptable carrier.

[0012] A gene therapy approach for treatment of mammals afflicted with bone tumors, such as Ewing's Sarcoma and osteosarcoma or expressing a tubedown-1 protein is provided. For this method of treatment, a biologically active antisense cDNA is generated from the cDNA of SEQ ID NO. 2 and administered to cells of an individual producing excess of a tubedown-1 gene.

[0013] This method further comprises in vivo administration into host cells a replicable vector comprising and expressing the desired antisense cDNA, which in turn produces the antisense mRNA. The vector is then

taken up by the cells to produce the antisense mRNA. This antisense mRNA binds to native mRNA produced by the tubedown-1 gene, thereby blocking expression of the gene. The antisense cDNA generates antisense mRNA of at least 70% complementarity to mRNA produced by a native tubedown-1 gene and which can hybridize with the native mRNA under low and high stringency conditions. The preferred antisense cDNA's for use in this gene therapy treatment are selected from the group consisting of SEQ ID NO. 3 and SEQ ID NO. 4 and mixtures thereof.

[0014]

In an alternative treatment for bone tumors, and Ewings Sarcoma family of tumors and osteosarcoma in particular, single-stranded antisense oligonucleotides derived from the antisense cDNA sequence of SEQ ID NO. 3 or 4 can be generated ex vivo. These antisense oligonucleotides are at least 15 nucleobases in length and preferably at least 25 nucleobases in length. Thus, when introduced into the cell, these antisense oligonucleotides cause inhibition of expression of tbdn-1 by hybridizing with native mRNA and genomic sequences of a tbdn-1 gene. Other biological or chemical factors to inhibit the expression of said tbdn-1 protein are also within the scope of this invention.

Brief Description of the Drawings

[0015]

Fig. 1: Tbdn-1 is highly expressed in developing bone and in bone tumors. Adjacent paraffin sections of day 14 gestation mouse embryo ribcage bones were stained using anti-tbdn-1 antibody Ab1272 (A) or preimmune IgY control antibody (B). Paraffin sections of primary human

osteosarcoma (C, 200X), primary Ewing's sarcoma (D, 200X; E, 400X) or xenografted human Ewing's tumor cell line EWS-96 (F, 400X) were stained using anti-tbdc-1 antibody or preimmune IgY control antibody (G, Ewing's xenograft, 400X; H, osteosarcoma, 200X). A, B, D, E, F and G were developed with alkaline phosphatase and fast red substrate (red stain) and counterstained with aqueous methyl green. C and H were developed with horseradish peroxidase (reddish-brown stain). Developing bone, both the primary bone tumors, and the xenografted Ewing's cell line showed intense tbdc-1 expression in tumor cells. Tbdn-1 staining was not seen in normal bone tissue (blue/green) surrounding the Ewing's sarcoma tumor (D) or was present at only very low levels in tissue surrounding the Ewing's xenograft (F, yellow-brownish areas, arrowed).

[0016] **Fig. 2:** The EWS/FLI-1 fusion protein is expressed in Ewing's sarcoma cell lines. Western blotting was performed on protein lysates of the indicated cell lines using an antibody to FLI-1 (14). FLI-1, which is expressed by the IEM embryonic endothelial cell line and both the EWS-96 and RD-ES Ewing's sarcoma cell lines, is indicated. The EWS/FLI-1 fusion protein, resolving as the larger molecular weight band indicated on the blot, is expressed only in the Ewing's sarcoma cell lines and not in the endothelial cell line.

[0017] **Fig. 3:** Tbdn-1 transcript and protein are highly expressed in Ewing's sarcoma cell lines. A, Northern blot analysis of RNA prepared from Ewing's sarcoma cell lines EWS-96, SJES8, SJES7 and SJES1 as

indicated, was performed using a tbdn-1 cDNA probe. B, Western blot analysis of protein lysates of embryonic and retinal endothelial cell lines (IEM and RF/6A, respectively) and Ewing's sarcoma cell lines (RD-ES and EWS-96) was performed using the anti-tbdn-1 antibody Ab1272 revealing the 69 kDa band corresponding to tbdn-1.

[0018] **Fig. 4:** Inhibition of tbdn-1 expression in Ewing's sarcoma cells using an antisense tbdn-1 construct. Anti-tbdn-1 (Ab1272) western blot analysis of EWS-96 Ewing's sarcoma cell clones stably over-expressing antisense tbdn-1 cDNA (AS_{tbdn-1}) show decreased expression of tbdn-1 protein (AS1, AS2 and AS3) compared to controls (parental, parental EWS-96 cells; vector, EWS-96 cells expressing the empty vector; AS control, EWS-96 cells transfected with AS_{tbdn-1} construct but showing no decrease in tbdn-1 expression).

[0019] **Fig. 5:** The antisense tbdn-1 construct AS_{tbdn-1} does not produce aberrant toxic proteins. In vitro protein translated from control (control), antisense tbdn-1 (AS_{tbdn-1}) or tbdn-1 (tbdn-1) constructs using [³⁵S] methionine/cysteine were separated on SDS-PAGE and analyzed by autoradiography. In control preparations, one major band of ~61 kDa is observed, while the tbdn-1 construct leads to synthesis of one major ~69 kDa band as expected. No protein is synthesized using the antisense tbdn-1 construct (AS_{tbdn-1}). Apparent molecular weight markers are indicated to the right of the gel.

[0020] **Fig. 6:** Inhibition of tbdn-1 expression suppresses Ewing's sarcoma tumor cell growth. Ewing's sarcoma cell (EWS-96) clones showing decreased expression of tbdn-1 (AS 2 and AS 3) exhibit reduced growth compared to controls (PAR, parental EWS-96 cells; Vector, EWS-96 cells expressing the empty vector; AS CTR, EWS-96 cells transfected with AS tbdn-1 construct but showing no decrease in tbdn-1 expression). Growth of EWS-96 cells were assayed in standard culture conditions (A), in soft agar (B), and as xenograft tumors (C). Values are expressed +/- SEM.

Detailed Description of the Invention

Definitions

[0021] As used herein, the term "*biologically active* " refers to a protein having structural, regulatory, or biochemical functions of a naturally occurring molecule.

[0022] "*Cells*," "*host cells*" or "*recombinant host cells*" are terms used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

[0023]

The term "*derivative*," as used herein, refers to the chemical modification of a polypeptide sequence, or a polynucleotide sequence. Chemical modifications of a polynucleotide sequence can include, for example, replacement of hydrogen by an alkyl, acyl, or amino group. A derivative polynucleotide encodes a polypeptide which retains at least one biological function of the natural molecule. A derivative polypeptide is one modified, for instance by glycosylation, or any other process which retains at least one biological function of the polypeptide from which it was derived.

[0024]

As used herein, the term "*gene*" or "*recombinant gene*" refers to a nucleic acid comprising an open reading frame encoding tbdn-1, including both exon and (optionally) intron sequences. A "*recombinant gene*" refers to nucleic acid encoding tbdn-1 and comprising tbdn-1 encoding exon sequences, though it may optionally include intron sequences which are either derived from a chromosomal tbdn-1 gene or from an unrelated chromosomal gene. The term "*intron*" refers to a DNA sequence present in a given tbdn-1 gene which is not translated into protein and is generally found between exons.

[0025]

The words "*insertion*" or "*addition*," as used herein, refer to changes in an amino acid or nucleotide sequence resulting in the addition of one or more amino acid residues or nucleotides, respectively, to the sequence found in the naturally occurring molecule.

[0026]

As used herein, the term "*nucleic acid*" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). This term is used interchangeably with the term "*oligonucleotide*." The term should also be understood to include, as equivalents, analogs of either RNA or DNA made from nucleotide analogs, and, as applicable to the embodiment being described, single-stranded (such as sense or antisense) and double-stranded polynucleotides.

[0027]

The phrases "*percent identity*" or "*percent homology*" refers to the percentage of sequence similarity found in homologues of a particular amino acid or nucleic acid sequence when comparing two or more of the amino acid or nucleic acid sequences.

[0028]

As used herein, the term "*transfection*" means the introduction of a nucleic acid, e.g., an expression vector, into a recipient cell by nucleic acid-mediated gene transfer.

[0029]

"*Transformation*", as used herein, refers to a process in which a cell's genotype is changed as a result of the cellular uptake of exogenous DNA or RNA, and, for example, the transformed cell expresses a recombinant form of tbdn-1, or where anti-sense expression occurs, from the transferred gene, the expression of a naturally-occurring form of tbdn-1 is disrupted.

[0030] The term "*tumor*" in the following specification denotes an uncontrolled growing mass of abnormal cells. This term includes both primary tumors, which may be benign or malignant, as well as secondary tumors, or metastases which have spread to other sites in the body.

[0031] As used herein, the term "*vector*" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. Preferred vectors are those capable of autonomous replication and/expression of nucleic acids to which they are linked. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which refer to circular double stranded DNA loops which, in their vector form are not bound to the chromosome. In the present specification, "plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto. Vectors may also be viral vectors wherein the viral vector is selected from the group consisting of a lentivirus, adenovirus, adeno-associated virus and virus-like vectors.

Discussion

[0032] The tbdn-1 protein (SEQ. ID. NO. 1) is highly expressed in developing bone and in bone tumors, including Ewing's sarcoma and osteosarcoma (Figure 1). Postnatal expression of tbdn-1 is restricted to the bone marrow and certain types of vasculature such as ocular and ovarian blood vessels (11). Tbdn-1 is downregulated during IEM capillary formation *in vitro*. Inhibition of tbdn-1 by expression of antisense tbdn-1 cDNA augments capillary formation of IEM cells.

[0033] The *in vivo* expression pattern of tbdn-1 apparently functions to regulate the growth and development of bone tissue. Tbdn-1 expression is not observed in normal postnatal bone cells. Accordingly, a recombinant antisense construct of tbdn-1 cDNA (tbdn-1 cDNA given by SEQ. ID. NO. 2) has been generated (tbdn-1 cDNA sequence in an antisense orientation) and expressed in both the IEM and bone tumor cell lines. It has been found that this recombinant antisense construct blocks expression of the tbdn-1 protein. Blockage of tbdn-1 expression in Ewing's sarcoma cells using an antisense tbdn-1 cDNA construct (ASTbdn-1) inhibits tumor growth *in vitro* and *in vivo* as xenografted tumors. These results are consistent with the premise that the tbdn-1 pathway controls growth of Ewing's sarcoma cells and that blockage of the tbdn-1 pathway provides a therapeutic alternative for treatment Ewing's tumors and also for osteosarcomas and bone tumors generally.

[0034]

Nucleic acids which have a sequence that differs from the nucleotide sequence shown in SEQ ID No. 2 due to degeneracy in the genetic code and having at least 70% sequence homology, are also within the scope of the invention. Such nucleic acids are functionally equivalent to tbdn-1 cDNA (i.e., a cDNA's having a biological activity equivalent to that of cDNA derived from tbdn-1 gene) but differ in sequence due to degeneracy in the genetic code. One skilled in the art will appreciate that these variations in one or more nucleotides of the nucleic acids encoding tbdn-1 protein may exist among individuals of a given species due to natural allelic variation. Any and all such nucleotide variations and resulting amino acid polymorphisms are within the scope of this invention.

[0035]

Fragments of the tbdn-1 cDNA sequence as well as the entire cDNA of tbdn-1 may be used to generate the antisense nucleotide sequences. The nucleic acid fragments include those capable of producing antisense cDNA constructs which form antisense mRNA's that hybridize under high or low stringency conditions with nucleic acids from other, including alternate isoforms, e.g. mRNA splicing variants.

[0036]

Antisense oligonucleotides derived from the cDNA of tbdn-1 include single-stranded antisense oligonucleotides that are at least 15 nucleobases in length and preferably at least 25 nucleobases in length. These antisense oligonucleotides hybridize with the native mRNA of tbdn-1 to block expression of tbdn-1. Such oligonucleotides within the scope of the invention may also contain linker sequences, modified restriction endonuclease sites and other sequences useful for molecular cloning,

expression or purification of recombinant forms of the subject tbdn-1 protein.

[0037] Antisense cDNA molecules include, but are not limited to base pairs of at least 1000 nucleobases, base pairs 1413-1 (SEQ ID NO. 3) and base pairs 3418-1 (SEQ ID NO. 4). The antisense cDNA's within the scope of the invention may also contain linker sequences, modified restriction endonuclease sites and other sequences useful for molecular cloning, expression or purification of recombinant forms of the subject tbdn-1 protein.

Methods of Treatment of Ewings Sarcoma, Osteosarcoma and Related Sarcomas

[0038] Invention primarily relates to the use of the isolated oligonucleotides in antisense therapy for the treatment of Ewing's Sarcoma, osteosarcoma and other or related sarcomas expressing high levels of tbdn-1. As used herein, *antisense* therapy refers to administration or *in situ* generation of antisense cDNA or oligonucleotides or their derivatives which specifically hybridize (e.g. binds) under low and high stringency conditions, with the native mRNA and/or genomic DNA encoding a tbdn-1 protein, so as to inhibit expression of that protein, e.g. by inhibiting transcription and/or translation. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix. In general, antisense therapy refers to the range

of techniques generally employed in the art, and includes any therapy, which relies on specific binding to oligonucleotide sequences.

[0039] Absolute complementarity, although preferred, is not required. A sequence *complementary* to a portion of an RNA, as referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex. In the case of double-stranded antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

[0040] An antisense construct of the present invention can be delivered, for example, as an expression plasmid which, when transcribed in the cell, produces RNA, which is complementary to at least a unique portion of the cellular mRNA which encodes a tbdn-1 protein.

[0041] Alternatively, the antisense construct can be an oligonucleotide, which is generated *ex vivo* and which, when introduced into the cell, causes inhibition of expression by hybridizing with the mRNA and/or genomic sequences of an tbdn-1 gene. Such oligonucleotides are preferably modified oligonucleotides, which are resistant to endogenous nucleases, e.g. exonucleases and/or endonucleases, and is therefore stable

in vivo. Exemplary nucleic acid molecules for use as antisense oligonucleotides are phosphoramidate, phosphothioate and methylphosphonate analogs of oligonucleotide (see also U.S. Pat. Nos. 5,176,996, 5,294,564 and 5,256,775, which are herein incorporated by reference). Additionally, general approaches to constructing oligomers useful in antisense therapy have been reviewed (13, 14).

[0042] Accordingly, the modified oligomers of the invention are useful in therapeutic, diagnostic, and research contexts. In therapeutic applications, the oligomers are utilized in a manner appropriate for antisense therapy in general. For such therapy, the oligomers of the invention can be formulated for a variety of loads of administration, including systemic and topical or localized administration. Techniques and formulations generally may be found in Remington's Pharmaceutical Sciences, Meade Publishing Co., Easton, Pa., and may include both human and veterinary formulations. For systemic administration, injection is preferred, including intramuscular, intravenous, intraperitoneal, and subcutaneous for injection, the oligomers of the invention can be formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the oligomers may be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included.

[0043] In clinical settings, the gene delivery systems for therapeutic tbdn-1 genes can be introduced into a patient (or non-human animal) by any of a number of methods, each of which is known in the art. For instance, a

pharmaceutical preparation of the gene delivery system can be introduced systemically, e.g. by intravenous injection, and specific transduction of the protein in the target cells occurs predominantly from specificity of transfection provided by the gene delivery vehicle, cell-type or tissue-type expression due to the transcriptional regulatory sequences controlling expression of the receptor gene, or a combination thereof.

[0044] The pharmaceutical preparation of the gene therapy construct can consist essentially of the gene delivery system in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery system can be produced intact from recombinant cells, e.g. retroviral vectors, the pharmaceutical preparation can comprise one or more cells which produce the gene delivery system.

[0045] Gene therapy methodologies can also be described by delivery site. Fundamental ways to deliver genes include *ex vivo* gene transfer, *in vivo* gene transfer and *in vitro* gene transfer. In *ex vivo* gene transfer, cells are taken from the patient and grown in cell culture. The DNA is transfected into the cells, and the transfected cells are expanded in number and then reimplanted in the patient. In *in vitro* gene transfer, the transformed cells are cells growing in culture, such as tissue culture cells, and not particular cells from a particular patient. These "laboratory cells" are transfected, and the transfected cells are selected and expanded for either implantation into a patient or for other uses. *In vivo* gene transfer involves introducing the DNA into the cells of the patient when the cells are within the patient. All

three of the broad based categories described above may be used to achieve gene transfer *in vivo*, *ex vivo* and *in vitro*.

[0046]

Mechanical (i.e., physical) methods of DNA delivery can be achieved by microinjection of DNA into germ or somatic cells, pneumatically delivered DNA-coated particles such as the gold particles used in a "gene gun" and inorganic chemical approaches such as calcium phosphate transfection. It has been found that physical injection of plasmid DNA into muscle cells yields a high percentage of cells which are transfected and have sustained marker genes. The plasmid DNA may or may not integrate into the genome of cells. Non-integration of the transfected DNA would allow the transfection and expression of gene product proteins in terminally differentiated, non-proliferative tissues for a prolonged period of time without fear of mutational insertions, deletions or alterations in the cellular or mitochondrial genome. Long-term, but not necessarily permanent, transfer of therapeutic genes into specific cells may provide treatments for genetic diseases or for prophylactic use. The DNA could be reinjected periodically to maintain the gene product level without mutations occurring in the genomes of the recipient cells. Non-integration of exogenous DNAs may allow for the presence of several different exogenous DNA constructs within one cell with all of the constructs expressing various gene products.

[0047]

Particle-mediated gene transfer may also be employed for injecting DNA into cells, tissues and organs. With a particle bombardment device, or "gene gun," a motive force is generated to accelerate DNA-coated high

density particles (such as gold or tungsten) to a high velocity that allows penetration of the target organs, tissues or cells. Electroporation for gene transfer uses an electrical current to make cells or tissues susceptible to electroporation-mediated gene transfer. A brief electric impulse with a given field strength is used to increase the permeability of a membrane in such a way that DNA molecules can penetrate into the cells. The techniques of particle-mediated gene transfer and electroporation are well known to those of ordinary skill in the art.

[0048]

Chemical methods of gene therapy involve carrier-mediated gene transfer through the use of fusogenic lipid vesicles such as liposomes or other vesicles for membrane fusion. A carrier harboring a DNA of interest can be conveniently introduced into body fluids or the bloodstream and then site-specifically directed to the target organ or tissue in the body. Cell or organ-specific DNA-carrying liposomes, for example, can be developed and the foreign DNA carried by the liposome absorbed by those specific cells. Injection of immunoliposomes that are targeted to a specific receptor on certain cells can be used as a convenient method of inserting the DNA into the cells bearing that receptor. Another carrier system that has been used is the asialoglycoprotein/polylysine conjugate system for carrying DNA to hepatocytes for in vivo gene transfer.

[0049]

Transfected DNA may also be complexed with other kinds of carriers so that the DNA is carried to the recipient cell and then deposited in the cytoplasm or in the nucleoplasm. DNA can be coupled to carrier

nuclear proteins in specifically engineered vesicle complexes and carried directly into the nucleus.

[0050]

Carrier mediated gene transfer may also involve the use of lipid-based compounds which are not liposomes. For example, lipofectins and cytofectins are lipid-based positive ions that bind to negatively charged DNA and form a complex that can ferry the DNA across a cell membrane. Another method of carrier mediated gene transfer involves receptor-based endocytosis. In this method, a ligand (specific to a cell surface receptor) is made to form a complex with a gene of interest and then injected into the bloodstream. Target cells that have the cell surface receptor will specifically bind the ligand and transport the ligand-DNA complex into the cell.

[0051]

Biological gene therapy methodologies employ viral vectors to insert genes into host cells. The host cells include, but are not limited to bacterial or eukaryotic cells. Viral vectors that have been used for gene therapy protocols include, but are not limited to, retroviruses, other RNA viruses such as poliovirus or Sindbis virus, adenovirus, adeno-associated virus, herpes viruses, SV 40, vaccinia and other DNA viruses. Replication-defective murine retroviral vectors are the most widely utilized gene transfer vectors. Murine leukemia retroviruses are composed of a single strand RNA completed with a nuclear core protein and polymerase (pol) enzymes encased by a protein core (gag) and surrounded by a glycoprotein envelope (env) that determines host range. The genomic structure of retroviruses include gag, pol, and env genes enclosed at the 5' and 3' long

terminal repeats (LTRs). Retroviral vector systems exploit the fact that a minimal vector containing the 5' and 3' LTRs and the packaging signal are sufficient to allow vector packaging and infection and integration into target cells providing that the viral structural proteins are supplied in trans in the packaging cell line.

[0052] Fundamental advantages of retroviral vectors for gene transfer include efficient infection and gene expression in most cell types, precise single copy vector integration into target cell chromosomal DNA and ease of manipulation of the retroviral genome. For example, altered retrovirus vectors have been used in ex vivo methods to introduce genes into peripheral and tumor-infiltrating lymphocytes, hepatocytes, epidermal cells, myocytes or other somatic cells (which may then be introduced into the patient to provide the gene product from the inserted DNA).

[0053] The adenovirus is composed of linear, double stranded DNA complexed with core proteins and surrounded with capsid proteins. Advances in molecular virology have led to the ability to exploit the biology of these organisms to create vectors capable of transducing novel genetic sequences into target cells in vivo. Adenoviral-based vectors will express gene product peptides at high levels. Adenoviral vectors have high efficiencies of infectivity, even with low titers of virus. Additionally, the virus is fully infective as a cell-free virion so injection of producer cell lines are not necessary. Another potential advantage to adenoviral vectors is the ability to achieve long term expression of heterologous genes in vivo.

[0054]

Viral vectors have also been used to insert genes into cells using *in vivo* protocols. To direct tissue-specific expression of foreign genes, cis-acting regulatory elements or promoters that are known to be tissue-specific may be used. Alternatively, this can be achieved using *in situ* delivery of DNA or viral vectors to specific anatomical sites *in vivo*. For example, gene transfer to blood vessels *in vivo* was achieved by implanting *in vitro* transduced endothelial cells in chosen sites on arterial walls. The virus-infected surrounding cells, in turn, also expressed the gene product. A viral vector can be delivered directly to the *in vivo* site (by catheter, for example) thus allowing only certain areas to be infected by the virus and providing long-term, site-specific gene expression. *In vivo* gene transfer using retrovirus vectors has also been demonstrated in mammary tissue and hepatic tissue by injection of the altered virus into blood vessels leading to the organs.

[0055]

The compounds of the present invention may also be useful for the prevention of metastases from the tumors described above either when used alone or in combination with radiotherapy and/or other chemotherapeutic treatments conventionally administered to patients for treating angiogenic diseases. For example, when used in the treatment of solid tumors, compounds of the present invention may be administered with chemotherapeutic agents such as alpha interferon, COMP (cyclophosphamide, vincristine, methotrexate and prednisone), etoposide, mBACOD (methotrexate, bleomycin, doxorubicin, cyclophosphamide, vincristine and dexamethasone), PROMACE/MOPP (prednisone,

methotrexate (w/leucovorin rescue), doxorubicin, cyclophosphamide, taxol, etoposide/mechlorethamine, vincristine, prednisone and procarbazine), vincristine, vinblastine, angiostatin, TNP-470, pentosan polysulfate, platelet factor 4, angiostatin, LM-609, SU-101, CM-101, Techgalan, thalidomide, SP-PG and the like. Other chemotherapeutic agents include alkylating agents such as nitrogen mustards including mechlorethamine, melphalan, chlorambucil, cyclophosphamide and ifosfamide; nitrosoureas including carmustine, lomustine, semustine and streptozocin; alkyl sulfonates including busulfan; triazines including dacarbazine; ethylenimines including thiotepa and hexamethylmelamine; folic acid analogs including methotrexate; pyrimidine analogues including 5-fluorouracil, cytosine arabinoside; purine analogs including 6-mercaptopurine and 6-thioguanine; antitumor antibiotics including actinomycin D; the anthracyclines including doxorubicin, bleomycin, mitomycin C and methramycin; hormones and hormone antagonists including tamoxifen and corticosteroids and miscellaneous agents including cisplatin and brequinar. For example, a tumor may be treated conventionally with surgery, radiation or chemotherapy and antisense cDNA tbdn-1 administration to stabilize and inhibit the growth of any residual primary tumor.

[0056]

When used in the above or other treatments, a therapeutically effective amount of one of the compounds of the present invention may be employed in pure form or, where such forms exist, in pharmaceutically acceptable salt form and with or without a pharmaceutically acceptable

excipient. A "therapeutically effective amount" of the compound of the invention means a sufficient amount of the compound to limit tumor growth or to slow or block tumor metastasis at a reasonable benefit/risk ratio applicable to any medical treatment. It will be understood, however, that the total daily usage of the compounds and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular patient will depend upon a variety of factors including the disorder being treated and the severity of the disorder; activity of the specific compound employed; the specific composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration; the route of administration; the rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific compound employed and like factors well known in the medical arts. For example, it is well within the skill of the art to start doses of the compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. If desired, the effective daily dose may be divided into multiple doses for purposes of administration. Consequently, single dose compositions may contain such amounts or submultiples thereof to make up the daily dose.

[0057]

Gene therapy also contemplates the production of a protein or polypeptide where the cell has been transformed with a genetic sequence

that turns off the naturally occurring gene encoding the protein, i.e., endogenous gene-activation techniques.

EXPERIMENTAL EXAMPLES

Cell Culture [0058]

The EWS-96 cell line is derived from a Ewing's sarcoma patient specimen and is obtained at diagnosis. This cell line is cloned using limiting dilution. EWS-96 cell line exhibits MIC2 surface expression as detected by flow cytometry analysis with the anti-MIC2 antibody O13 (Signet Laboratories, Dedham MA.). The t(11;22) fusion mRNA product is detected in this cell line by RT-PCR. All experiments involving the use of pathological specimens of human tissues in these studies are obtained under informed consent under the approval of the Institutional Review Board of Children's Hospital Medical Center (Cincinnati, OH). All research on human specimens follows the tenets of the Declaration of Helsinki at all times. RD-ES and RF/6A cells are obtained from the American Type Culture Collection (Manassas, VA), while the IEM cell line has been previously described (15). SJES 1, 7, and 8 Ewing's sarcoma cell lines were kindly provided by Dr. Thomas Look at St. Jude Children's Research Hospital (Memphis, TN). All cell lines are grown and maintained in culture in low glucose Dulbecco's Modified Eagle Media (DMEM) supplemented with 2 mM glutamine, and 10 % fetal bovine serum (FBS). For RF/6A cells, culture media is further supplemented with 50 uM of non-essential amino acids.

[0059] EWS-96 cells are transfected by lipofection with the vector pcDNA3.1/Zeo (Invitrogen, CA) alone, or with a construct of the pcDNA3.1/Zeo vector harboring *tbdn-1* cDNA nucleotide sequences 1-1413 in an antisense orientation (AStbdn-1). Two days after transfection, cells are selected with 25 ug/ml of Zeocin (Invitrogen, CA). After selection, clones are picked with cloning cylinders, expanded and characterized.

Xenograft tumors

[0060] For Ewing's sarcoma xenograft tumors, *NOD scid B2m* mice (Jackson Laboratory, Bar Harbor ME) are injected subcutaneously with 5×10^6 viable EWS-96 cells (parental or transfected). Cell viability is assessed by trypan blue dye exclusion. Tumor growth of EWS-96 cells is monitored on a daily to weekly basis by measurement using fine calipers. After sacrifice, tumors are carefully dissected and weighed immediately thereafter. Xenograft human osteosarcoma tumors are obtained from the *nu/nu* mice xenograft bank of the Division of Hematology/Oncology at Children's Hospital Medical Center (Cincinnati, OH). Origin of the xenograft osteosarcomas is confirmed by chromosome analysis. All experiments involving animals are performed in accordance with the National Institutes of Health Guidelines on the Care and Use of Laboratory Animals and are approved by the Children's Hospital Medical Center (Cincinnati, OH) Institutional Animal Care and use Committee.

Western Blotting

[0061] Cell lysates are prepared using Triton-X 100 lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton-X 100) supplemented with protease inhibitors (1 mM PMSF, 0.3 U/ml aprotinin, and 10 ug/ml leupeptin) and phosphatase inhibitors (1 mM sodium orthovanadate, 25 mM sodium fluoride, and 10 mM betaglycerophosphate. Lysates are clarified by centrifugation and protein is quantified and analyzed by SDS-PAGE. Gels are processed for western blotting using antibodies directed against FLI-1 (Santa Cruz Biotechnology, CA) and *tbdn-1* (Ab1272, ref. 22). Incubations, washes and western blotting are performed by standard procedures using chemiluminescence detection (ECL Plus reagent, Amersham).

Northern blotting

[0062] Northern blotting is performed as previously described (Sambrook et al., 1989). Blots are hybridized with a ³²P-labeled 5'end fragment of the *tbdn-1* cDNA (nucleotides 1-1413) as probe. Blots are reprobbed with a mouse 18S ribosomal RNA cDNA in order to confirm loading equivalency and RNA integrity.

Immunocytochemistry

[0063] Immunocytochemistry is performed on paraformaldehyde fixed, paraffin embedded sections of mouse and human tissue specimens to detect *tbdn-1*. Some sections are incubated in a solution of

methanol/peroxide to quench endogenous peroxidase activity in cases of peroxidase substrate reactions. Following a 1 hour blocking step in 2% normal goat serum, sections are incubated with either a 1/100 dilution of anti-tbdcn-1 antibody Ab1272 or preimmune IgY. After rinsing in PBS, reactions are developed using the appropriate peroxidase or alkaline phosphatase conjugated species specific secondary antibodies (Promega, Madison, WI). Red color reactions are generated using amino ethylcarbazole substrate (AEC, Sigma) in the presence of H₂O₂ for peroxidase reactions or naphthol-AS-MX Phosphate in the presence of Fast Red and Levamisole (to block endogenous tissue alkaline phosphatase activity) for alkaline phosphatase reactions. Peroxidase reacted sections are mounted in Cytoseal-60 (Stephens Scientific). Alkaline phosphatase reacted sections are counterstained using a 0.5% aqueous solution of methyl green, rinsed in water, dried and finally mounted in Permount (Fisher, Pittsburgh, PA). Sections are viewed and photographed using a Nikon microscope system with a Kodak DC120 digital camera attachment.

In vitro transcription and translation

[0064] In vitro transcription and translation of the various constructs with the T7 TNT-coupled rabbit reticulocyte lysate system (Promega) are carried out according to the manufacturer's procedure in the presence of [³⁵S]methionine/[³⁵S]cysteine (1175 Ci/mmol; NEN Life Science Products, Boston, MA). Samples are analyzed by SDS-PAGE and autoradiography.

In vitro growth assays

[0065] Parental and transfected EWS-96 cell clones are plated at 1×10^5 , 2.5×10^5 and 5×10^5 cells per 35mm dish in standard culture media and cultured for 72, 48 and 24 hours, respectively. After culturing, cells are trypsinized and the number of viable cells per dish is determined in triplicate samples using trypan blue dye exclusion. For growth in soft agar, 2×10^5 parental or transfected EWS-96 cells are plated into a 60 mm dish in 2 ml of 0.33% Agar Noble (Difco Laboratories) in complete culture media over a layer of 2 ml of 0.5% Agar Noble in complete media. Between 7 to 10 days after plating, colonies growing in soft agar are counted.

Results

[0066] The EWS-96 cell line expresses the hallmark EWS/FLI-1 fusion protein (Fig. 2) characteristic of Ewing's sarcoma. The EWS/FLI-1 fusion protein expressed by the EWS-96 cell line co-migrates with the EWS/FLI-1 type II fusion protein expressed by the Ewing's sarcoma cell line RD-ES (Fig. 2). All Ewing's sarcoma cell lines examined are positive for tbdn-1 transcript and/or protein (Fig. 3).

[0067] To address the functional role of tbdn-1 in Ewing's sarcoma, clones of Ewing's sarcoma cells are generated by stably over-expressing antisense tbdn-1 cDNA fragment (AS_{tbdn-1}) in order to block tbdn-1 expression. The AS_{tbdn-1} construct does not encode an irrelevant protein product which could be nonspecifically toxic to the Ewing's sarcoma cells (Fig. 4).

Western blot analysis revealed that several Ewing's sarcoma cell clones transfected with the AStbdn-1 construct exhibited reduced expression of tbdn-1 protein expression when compared to the parental cells or Ewing's sarcoma cell clones expressing the empty vector (Fig. 5, and data not shown).

[0068] The effect of downregulation of tbdn-1 expression was examined on both *in vitro* and *in vivo* growth of the Ewing's sarcoma cells. The *in vitro* growth (under standard culture conditions) and the anchorage independent growth in soft agar of clones of Ewing's sarcoma cells exhibiting reduced levels of tbdn-1 expression is significantly reduced compared to control cells (parental cells, cells transfected with the vector alone or with the AStbdn-1 construct but showing no reduction in tbdn-1 levels [Fig. 5]) (Fig. 6A and B, respectively).

[0069] Furthermore, the downregulation of tbdn-1 expression in Ewing's sarcoma cells greatly inhibits their growth as tumors *in vivo* in a xenograft model compared to the control clones (Fig. 6C). The levels of tbdn-1 expressed by the different EWS-96 clones (parental, transfected with the vector alone or AStbdn-1) correlates with their rate of growth in soft agar and as xenografts (Fig. 5, 6B and C). EWS-96 clone AS3 which expresses the lowest level of tbdn-1 (Fig. 5) exhibited the most reduced growth rate in soft agar and as xenograft tumors (Fig. 6B and C). EWS-96 clone AS2, which expresses intermediate levels of tbdn-1 (more than clone AS3 but less than controls, Fig. 5) exhibits intermediate growth rates (higher than

clone AS3 but less than controls) in soft agar and as xenograft tumors (Fig. 6B and C).

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SEQUENCE LISTINGS

(1) General Information

(iii) NUMBER OF SEQUENCES: 4

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 593 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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Arg	Gly	Leu	Val	Pro	Arg	Lys	Leu	Pro	Leu	Asn	Phe	Leu	Ser	Gly	Glu	20	25	30	
Lys	Phe	Lys	Glu	Cys	Leu	Asp	Arg	Phe	Leu	Arg	Met	Asn	Phe	Ser	Lys	35	40	45	
Gly	Cys	Pro	Pro	Val	Phe	Asn	Thr	Leu	Arg	Ser	Leu	Tyr	Arg	Asp	Lys	50	55	60	
Glu	Lys	Val	Ala	Ile	Val	Glu	Glu	Leu	Val	Val	Gly	Tyr	Glu	Thr	Ser	65	70	75	80
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Pro	Pro	Thr	Thr	Leu	Leu	Trp	Val	Gln	Tyr	Tyr	Leu	Ala	Gln	His	Tyr	100	105	110	
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Ile	Glu	Ser	Thr	Pro	Thr	Leu	Ile	Glu	Leu	Phe	Leu	Val	Lys	Ala	Lys	130	135	140	
Ile	Tyr	Lys	His	Ala	Gly	Asn	Ile	Lys	Glu	Ala	Ala	Arg	Trp	Met	Asp	145	150	155	160
Glu	Ala	Gln	Ala	Leu	Asp	Thr	Ala	Asp	Arg	Phe	Ile	Asn	Ser	Lys	Cys	165	170	175	
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Arg	His	Phe	Ile	Glu	Ile	Thr	Asp	Asp	Gln	Phe	Asp	Phe	His	Thr	Tyr	245	250	255	
Cys	Met	Arg	Lys	Ile	Thr	Leu	Arg	Ser	Tyr	Val	Asp	Leu	Leu	Lys	Leu	260	265	270	
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Asn	Lys	Glu	His	Glu	Ala	Asp	Thr	Ala	Asn	Met	Ser	Asp	Lys	Glu	Leu	305	310	315	320
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Phe	Leu	Lys	Arg	Asn	Ser	Asp	Ser	Leu	Pro	His	Arg	Leu	Ser	Ala	Ala	485	490	495	
Lys	Met	Val	Tyr	Tyr	Leu	Asp	Ser	Ser	Ser	Gln	Lys	Arg	Ala	Ile	Glu	500	505	510	
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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 3418 base pairs
- (B) TYPE: cDNA
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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 AGTACTATTT GGCACAGCAT TATGATAAAA TTGGTCAGCC ATCCATTGCT CTGGAATACA 780
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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 1413 base pairs

(B) TYPE: cDNA

(C) STRANDEDNESS: both

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 3418 base pairs

(B) TYPE: cDNA

(C) STRANDEDNESS: both

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CCCAAAACAA TTTATTGCAT ATTTGGTACC TTATGTTTGA CAACAAAAAA AAAAAGAAAT 60
 TACATTTGTA ACTGCAGGAA AAAGAAGCTG GTCTGTTATT GAAAAAATAA TCTTGGGCCC 120
 TTTAAAAATT ACAAAGTCTT GGGCTTGCCT GAAACAACTG AGCGAGAAAT ATATTCTTAG 180
 AAATGAAGGG CTTCAAGTAT TACAAACCTG TGACACTGTG GAAAAGTTCC CAGAGGTATC 240
 TAAACTGCAG CACTTTTTCT GCATATCGTG AACAAGGCCT GCTGAGGTTT TAGATATTAG 300
 TCTCTTCATC AGTCATAGAA TGGTAGTCAG GAGTTCTGCT CCTGTAACCT CAAGCCATGG 360
 TAGAGTTTTT TTTTTTCACT CTCAGGTGAA AAATTCATGC TTGCATAATT AGTTGGTGCC 420
 ACATCACAGT GCATTTGGTT CTTGCATTAC AGTTTAAACA AGTTTGGCCA AAACAATGAT 480
 AAAGGTTTGC ATTGTTATTT TAGTTCTTCA AGTTTATAGT AAATTGAGAT TGTTCCAAC 540
 GGTTCCTTCG GTTAAAAACT GTGGTACAAG AACACCAAAC TGATCATGTA CAGTGAATTT 600

TGGAACACA ACACACGTAC TGAACACCTT CTAGGTTTCA CTTAATCCGC TTGGTAGCTA	660
TGTGTCCCAG TCTCTACAAC GTCAAGCATG TGTGTGTAGAC GGCTCTGTAC ACAGTGATTTC	720
ACATTGCATA CTTCTATG TCAATCACCA ACCCCGTAAC AGAATCAGGC AGATGCTAAA	780
GCCCCAACAA CTCTTTGTGC TCAGTGAAAG AATCCAGTGC TAAAACAGCA TTTATTTATG	840
CTGTCTGAGA TATAGCAAAA TCTCACAAGA CAAATTTAAA GTAATATTTT TGCCACACAC	900
CTGCTATGTT AGCTGTTATA GCCTCCAGAT TTTATATAAA TTAGTTAAAA ACATGGGAAG	960
GGAAGGTAGG AATAGGATAC GGATTTTTTT TTCTTTTAAA CCCCTCAATT TTAGCAGCTT	1020
TTAATTTTTT AAGAACTGA ACCTATATCC TGTAATGTTA GATATTTTAT ATATAGTTTT	1080
CAGCAGGATA AAAAACGTAA GACTATTTGA AGGCAAGAAC ATTTACTCCT CTCATTCTGT	1140
GTAAGTAAGA GCAATGCAGC AGGTGCGTGA CAAAAATATT ATACACTAGA TATGGTCCAA	1200
AGTCATTCCA TTTGCTTGTT TAATGATGTT CAGATTTTCA TGGCCAGTTC TTCCGTTTCT	1260
GCAGAACTAT CTCCGTTTAC TGTGATCTTC ATATCCTCTT CGTATCCAGG AGGCATGAAA	1320
GCCAAAGCAT AAGGGAAAAG CTATGACAA CTTGCTCTGT AGGCTTCGGC AGCTTCTTTA	1380
CAGTCTCGTA GGCTACCATC ACACAAGGCT TCCAACACTT CCATGCAAGT CTGAAGGTTT	1440
CTGTTGGTGA GGGATCCATC AAGTGTGTG GCCAGCTCTA TTGCTCGTTT TTGACTAGAA	1500
GAATCTAAAT AATATACCAT TTTGGCAGCT GATAATCTAT GTGGCAATGA ATCAGAATTC	1560
CTTTTCAGAA AGGTTTCATT AAAATCTTTT GGATTTGTTG CTCCAAAAAG ACGATTCATT	1620
TCTTGTTTTA ATACTGTTCT AACTGTTTCG GGTAAGTCTT TACTTTCACA CACAGAATGA	1680
AAGAGTCGAA TCATGCACTC ATGAAGCCAG GGATGACTAG AATCAATAGC AAATGCCCGC	1740
TTTACTGATT GTAGCATCAA AAGAACTTTT TCTTTCCTAA AGTAGATCTC AAAGGCAAAA	1800
AGATGAGTTT CTATCTTGTT CTTACCAAG TTCTTCAATG GTGTTAAAAA CTTAATAGCT	1860
TCTTCCAATG GAGTTTCAAC CTTGGCCAGT TTCTCAGGGA TAAGCTCTTC TTTGGGGCCT	1920
CCAATTTCTT CGTCATCATC ATCCTTTTTT TTTTTCGGAT TCCGTTGCGG CTTTTCTTTT	1980
TCGGCATTTT TTTTCTCTTC TTCAATCTGG GCTTCTTTT GAGCTCTTCT TTGTTTATTA	2040
CGCAGTTTCT TTAGCTCTTT GTCAGACATG TTTGCTGTAT CAGCCTCGTG TTCTTTGTTC	2100
TCATCTGTCA GAGGGTTGTC ATGAAGCTTC AAATAGATCT CAATAGCAAT TCTCGCTGCT	2160
TTGAAGTAAA ATGGATGCTG TCGAAGTACA TCTTCTAGTT TTAATAAGTC CACATATGAT	2220
CTAAGGGTGA TCTTCCTCAT ACAGTATGTA TGAAAGTCAA ACTGGTCATC GGTGATTCTT	2280
ATAAAATGTC TCTCAATTTC ATGACATTTT TTAAGTGCTT CACCAAATT GTTCATTGCT	2340
TTGTATGCCT GAGCACACTC TGTCTGGAAC CACATACACT GCATTTTCATT CAGGTTCTCT	2400
ACCGCTGAAG TTCCTTCCCT CGTAACTTG GAACACATTT CTTCAGCCTC TTTAATCAGG	2460
TTGGCTTTTA ACATGTATTT TGCACACTTG GAATTAATAA ATCTGTCTGC TGTGTCCAGG	2520
GCCTGGGCTT CATCCATCCA CCTGGCAGCT TCTTTAATAT TCCCAGCATG CTTATAGATT	2580

TTAGCTTTTA	CAAGAAAGAG	TTCTATCAAT	GTTGGTGTAC	TTTCAATTGC	AGTATTTATG	2640
TATTCAGAG	CAATGGATGG	CTGACCAATT	TTATCATAAT	GCTGTGCCAA	ATAGTACTGG	2700
ACCCAAAGTA	ATGTGGTTGG	AGGTTCCCTCC	TTTCCATCAT	CATTGGGGTT	AAATAGGCGA	2760
CAACTTTTTA	GAGAAGTTTC	ATAACCAACT	ACTAGTTCTT	CTACGATTGC	CACCTTCTCT	2820
TTATCTCTGT	ATAAAGACCT	CAAGGTATTG	AAGACAGGTG	GACAGCCCTT	GCTGAAATTC	2880
ATCCTTAGGA	ACCTATCCAA	AACTCCTTA	AACTTCTCTC	CAGATAAAAA	GTTTAAGGGC	2940
AGCTTTCTTG	GCACGAGTCC	CCTGGGGTAT	TTAGTCCAGG	CTTCCTCATA	TATTTTTAGC	3000
CGTTCTAACA	TATTAGCTGG	CTTCAGTGCT	TTTTCTAAGC	CTTTGTAATA	GGCCCAATTT	3060
TCAGGATTCC	TCTCTTGTA	TCCTCTATA	ACGTCAGCAG	CATCTTCCAA	ACGACACAAC	3120
TGCAACAGAA	GTTCCCCTTT	GGTTTCTTCA	ACAGCAAGTT	TATCACAAAT	CTGCTTTTCA	3180
TAGGTACAAA	GATGTTCCAG	GGCTTCTCTA	TAAAGACCTG	CTTCCCGAAG	AACTTGATTC	3240
TGATATAAGA	GGAGTTCAC	ATATTCATA	TCCACTTTAT	CAGGAGATGT	CTGCTGTGTT	3300
TTCTTAAACT	CTTCTAAAAT	TTTTGCTGCC	ATTTCATAGT	CTTCTAATAA	ATGGTAAAGC	3360
ATAACCAATC	CATGATGCTC	ACTCTGCAGA	TCCTCTATCA	TCTTGCGGGT	GTTACTTG	3418